Analysis and expression of the polyhedrin gene of Antheraea pernyi nucleopolyhedrovirus (AnpeNPV)

Jia-Xi Huang¹, Hui-Ling Wu¹, Yan Wu¹, Shan-Ying Zhu¹, Wen-Bing Wang¹

Institute of Life Sciences, Jiangsu University, 301 Xuefu Road, Zhenjiang 212013, P. R. China

Received Dec. 3rd, 2008; revised Jan. 1st, 2009; accepted Jan. 5th, 2009

ABSTRACT

The polyhedrin (polh) gene is often used to analyse evolution of baculovirus. In this report, the polh of Antheraea pernyi nucleopolyhedrovirus (AnpeNPV) was cloned and sequenced. The Open reading frame (ORF) of the AnpeNPV consists of 738 nucleotides encoding 245 amino acids with molecular masses of 29 kDa. The deduced amino acids were significant homology with other baculoviruses, such as Attacus ricini NPV (ArNPV) and Autographa californica NPV (AcNPV). A strongly hydrophilic region was predicted at positions from 30 to 50 of the AnpeNPV Polh protein by bioinformatics analysis. Expression of the polh gene of AnpeNPV in E. coli was examined by SDS-PAGE, Western blot and Mass-spectrum analysis. The result showed that the bacterium expression system was suitable for the virus gene expression. It indicated that the products of the polh gene expressed in this system can be easier to use for raising antibodies.

Keywords: Antheraea Pernyi, Insect, Baculovirus, NPV, Polyhedrin, Prokaryotic Expression

1. INTRODUCTION

The Chinese oak silkworm Antheraea pernyi (Lepidoptera: Saturniidae) is an economically important insect primarily for the production of tussah silk. In recent years, consumption of the silkworm pupae as food delicacies has also gained tremendous popularity. The jaundice disease of the oak silkworm caused by the infection of A.pernyi nucleopolyhedrovirus (AnpeNPV) is a major threat to the tussah industry [1]. AnpeNPV is a member of the Baculoviridae with large, enveloped, double-stranded DNA. Baculoviridae are widely known to the scientific community in the form of commercial baculovirus expression vectors (BEVs) [2,3]. Baculoviruses also have an established application as insecticides against agricultural and forestry pests [4,5]. Currently, the Baculoviridae comprises two genera, Nucleopolyhedrovirus (NPV) and Granulovirus (GV) [1]. During the infection cycle, NPVs produce two structurally and functionally distinct virion phenotypes: occlusion- derived virus (ODV) and budded virus (BV) [6]. The occluded viruses of the NPV are referred to as polyhedra. Polyhedrin is the major protein component of the polyhedra [7]. The polh gene is not essential for viral development, and normally deletion of the polh gene is not interfering with viral replication in cultured cells. However, in per os infectivity, the polyhedra or occlusion bodies are required for the oral infection of insects [8]. Baculovirus entry into host cells involves that ODVs are released from the occlusion body by the alkaline environment within the midgut lumen of the larva and subsequently initiate primary infection of the mature columnar epithelial cells of the midgut [6].

In order to explore effective propagation and infectivity of the polyhedra, this paper analysised the nucleotide sequence and promoter (prmoter-Ap) of the polh gene of AnpeNPV by bioinformatics tools, and further prokaryotic expression for AnpeNPV polyhedrin (polh-Ap).

2. MATERIALS AND METHODS

2.1 Materials

The Wild-type AnpeNPV strain was maintained in our laboratory. Restriction Enzymes, T4 DNA ligase, PCR reagents pMD18-T and DNA purification kit were purchased from TaKaRa Company (China, Dalian); primers and other reagents were bought from Shanghai Sangon Bio-technology Corpotation. The vectors for expression, and *Escherichia coli* strain DH5α and BL21 were kept in our laboratory.

2.2. Aplification of the AnpeNPV polh Gene

AnpeNPV genomic DNA was isolated using the method described by previously [9,10] and about 15-20 ng DNA was used as template for standard PCR. The specific primers were designed based on the sequence of ORF (GenBank: EU195295). The polh-Ap forward primer (5' CCG <u>GAA TTC</u> ATG CCA GAT TAC TCA TAC CGG 3')containing an *Eco*R I restriction site (underlined), and the reverse primer (5' CCC <u>AAG CTT</u> CTA GTA CGC GGG GCC AGT 3') containing a *Hind* III restriction site (underlined). The PCR conditions were 1 cycle at 94 °C for 5 min; 30 cycles at 94 °C for 45 s, 62°C for 45 s, and 72 °C for 1 min; and 1 cycle at 72 °C for 10 min. The PCR product was examined by electrophoresis in 1% agarose gel with the ethidium bromide staining.



129

2.3. Cloning and Construction of Expression Plasmid

The PCR products were ligated into pMD18-T vector using T4 DNA ligase and then transformed into *E. coli* (DH5a), and sequenced, respectively.

The recombinant plasmid pMD-polh-Ap was digested with *Eco*R I and *Hind* III, and was purified to ligate with the Pet28a vector digested with *Eco*R I and *Hind* III, and transformed into *E. coli* (BL21).

2.4. Analysis of the polh Gene

The amino acid sequence was deduced with Expasy Translate tool (http://au. expasy. org/tools/dna. html) according to the AnpeNPV *polh* gene sequence. Align using DNAstar CLUSTAL W program. Phylogenetic tree was made by MEGA 3.1 software.

In order to explore regulatory sequence in the putative promoter region, NNPP (Promoter Predication by Neural Network http://www.fruitfly.org/seq_tools/promoter.html), promoter scan and transcription factor binding sites (http://www-bimas.cit.nih.gov/molbio/proscan/) were applied together to make a comprehensive prediction.

2.5. Expression of the polh Gene in E.coli

A positive clone was cultured in LB medium supplement with Kanamycin (final concentration of $50\mu g/ml$) ovenight at 37 °C with shaking, then the culture was added into 100mL fresh LB medium and cultured at 37°C with shaking to A600 about 0.6. The culture was induced with IPTG (final concentration of 8 µg/ mL) and shaked at 30 °C for 10 hours. SDS polyacrylamide gel was used to analyze the expression in the Mini-Protein system (Bio-Rad, USA). After electrophoresis, the gel was stained with Coomassie Brilliant Blue R250 to visualize the protein bands. Protein samples were separated on SDS-10% polyacrylamide gels and transferred to PVDF membranes. Blots were soaked in TBST buffer (10 mmol/L Tris-HCl, pH 7.6, 0.15 mol/L NaCl, 0.1% Tween 20) with 5% nonfat dried milk. The antiserum against the His-Polh fusion protein (His antibody) at a dilution of 1:2,000 monoclonal antibody was added as the first antibody, followed by addition of 1:5,000 dilution horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G as the secondary antibody. Blots were visualized with the Enhanced chemiluminescence Western blot kit (Amersham). The predicted Polh protein band was cut out for Mass-spectrum analysis.

3. RESULTS

3.1. Nucleotide and Amino Acid Sequence Analysis

The ORF of cloned gene has two different nucleotides from the published sequence (DQ486030), but no amino acid residues were changed. The 738 nucleotides (including the stop codon TAG) encoded a putative peptide of 235 amino acids by an Expasy Translate tool.

ATGCCAGATTACTCATACCGGCCGACCATTGGTCGCACCTATGTGTACGACAACAAGTAT M P D Y S Y R P T I G R T Y V Y D N K Y TACAAAAACTTAGGGTCCGTCATTAAAAACGCCAAGCGCAAGAAGCATTTAGTCGAACAT YKNLGSVIKNAKRKKHLVEH GAAGAGGAAGAAAAGCATTGGGATCCTTTAGACAATTACATGGTCGCGGAAGACCCTTTC E E E K H W D P L D N Y M V A E D P F E CTGGGGCCGGGTAAAAACCAAAAACTGACACTTTTCAAGGAAATCCGCAACGTTAAACCC L G P G K N Q K L T L F K E I R N V K P GACACAATGAAACTTATTGTCAACTGGAGCGGTAAAGAATTTCTGCGCGAAACTTGGACC D T M K L I V N W S G K E F L R E T W Т CGTTTTGTTGAGGATAGCTTTCCGATTGTAAACGACCAAGAGGTCATGGATGTGTTCCTC R F V E D S F P I V N D Q E V M D V F L GTCATTAACCTGCGCCCCACGCGCCCCAACAGGTGCTACAAGTTCCTGGCGCAGCACGCG I N L R P T R P N R C Y K F L A Q H A V CTCAGATGGGACTGCGACTACGTGCCGCACGAGGTAATCCGCATTGTGGAGCCATCCTAC R W D C D Y V P H E V I R I V E P S L Y GTGGGCATGAACAACGAGTACAGAATTAGCCTCGCCAAGAAAGGCGGCGGCTGCCCCATC G M N N E Y R I S L A K K G G G C P V Τ ATGAACATTCACAGCGAGTACACCAACTCGTTTGAATCGTTTGTAAACCGCGTAATCTGG M N I H S E Y T N S F E S F V N R V I GAGAACTTTTACAAGCCCATTGTGTACATTGGCACGGACTCGGGTGAGGAGGAGGAAGAATT ENFYKPIVYIGTDSGEEEEI CTCATCGAGGTTTCGCTTGTGTTCAAGGTCAAGGAGTTTGCGCCCGACGCGCCACTGTTT LIEVSLVFKVKEFAPDAPLF ACTGGCCCCGCGTACTAG Т G P A Y

Figure 1. Nucleotide sequence and deduced amino acid sequence of the polyhedrin gene. The predicted amino acid is represented by the one letter code designation below the nucleotide sequence. The initiate and the stop codes are framed.

The nucleotide sequence of Polh-Ap and its deduced amino acid sequence are shown in **Figure 1**. This deduced polypeptide contains 16 strongly basic, 16 strongly acidic, 113 hydrophobic and 58 hydrophilic amino acids with the calculated molecular mass of 29 kDa, and the isoelectric point was of 6.1.

3.2. Protein and Homology Analysis

Using BLAST software of NCBI to search for homology in the GenBank database, the deduced amino acid sequence showed an identity of 97%, 98%, 98%, 97%, 93% and 89% to the corresponding genes of *Attacus* ricini NPV(ArNPV, AAP16625), Epiphyas postvittana NPV (EppoNPV, NP_203170), Maruca vitrata MNPV (YP_950731), Rachiplusia ou MNPV (RoMNPV, NP_702998) [11], Bombyx mori NPV (BmNPV, AAA 46734) [12] and Autographa californica NPV (AcNPV, NP_054037) [13], respectively. Comparison of the deduced amino acid sequence with that of the corresponding genes of many species is shown in Figure 2. This protein was demonstrated to be highly conserved in baculoviruses.

The predict of secondary structure for polh-Ap by CLC Protein Workbench 3.0.3.(Figure 4). There are 4 regions

		*	20	*	40	*		
AcMNPV.Pro	:	MPD-YSYRPTI	GRTYVYDNKYY	KNLG <mark>AVIKNAKRE</mark>	KHFAEHEIE	FATIDE	:	49
AgseNPV.Pr	:	MYTRYSYNPHV	GRTYVYDNKFY	KNLGSVIKNAKR	EHLICHEIE	EKSLDE	:	50
AngeMNPV.P	:	MPD-YSYRPT1	GRTYVYDNKYY	KNLG <mark>S</mark> VIKNAKRE	KHLLEHQEE	EKSLDG	:	49
AnpeMNPV.P	:	MPD-YSYRPT1	GRTYVYDNKYY	KNLGSVIKNAKRE	KHLVEHDEE	EKHWDE	:	49
ArMNPV.Pro	:	MPD-YSYRPTI	GRTYVYDNKYY	KNLG <mark>S</mark> VIKNAR <mark>A</mark> F	KHLVEHDEE	EKHWDP	:	49
EmNPV.Pro	:	MPN-YSYTPT1	GRTYVYDNKYY	KNLGCLIKNAKR	KHLVEHDQE	BKQWDL	:	49
BusNPV.Pro	:	MYTRYSYKPSI	GRTYVYDNKYY	KNLG <mark>A</mark> VIKNAKRE	KHEIEHEVE	ERTIDE	:	50
CfMNPV.Pro	:	MPD-YSYRPT1	GRTYVYDNKYY	KNLGSVIK <mark>R-</mark> KRF	KHLLEHDED	EKHLDP	:	48
ChchNPV.Pr	:	MYTRYSYNPSI	GRTYVYDNKYY	KNLG <mark>AVIKNAKRE</mark>	KHYAEHDLE	EKELDP	:	50
Choristone	:	MPD-YSYRPT1	GRTYVYDNKYY	KNLGSVIKNAKRE	KHLLEHDED	EKHLDE	:	49
EcobNPV.Pr	:	MYTRYSYNPSI	GRTYVYDNKYB	KNLG <mark>A</mark> VIKNAKRE	KHQLEHEVE	EHALDE	:	50
EppoNPV.Pr	:	MPD-YSYRPT1	GRTYVYDNKYY	KNLG <mark>S</mark> VIKNAKRF	KHLLEHDED	EKHLDP	:	49
HearNPV.Pr	:	MYTRYSYSPTI	GKTYVYDNKYB	KNLG <mark>A</mark> VIKNAKRE	KHLEEHDHE	ERNIDS	:	50
HycuNPV.Pr	:	MPD-YSYRPT1	GRTYVYDNKYY	KNLG <mark>A</mark> VIKNAKRF	KHFAEHDIE	B ATIDE	:	49
LdMNPV.Pro	:	MHNFYNYSPAI	GKTYVYDNKYY	KNLGUVIKÇAKRÇ	KHLEQHE IE	D RSIDH	:	50
MacoNPV-A.	:	MYTRYSYNPSI	GRTYVYDNKYY	KNLG <mark>AVIKNA</mark> NRI	KHFIDHDLD	EKTLDE	:	50
MacoNPV-B.	:	MYTRYSYNPSI	GRTYVYDNKYY	KNLG <mark>S</mark> VIKNA <mark>N</mark> RF	KHYIEHDLE	EKTIDP	:	50
Maruca vit	:	MPD-YSYRPTV	GRTYVYDNKYY	KNLGSVIKNAKR	KHLVEHDEE	EKHWDE	:	49
OpMNPV.Pro	:	MPD-YSYRPT1	GRTYVYDNKYY	KNLG <mark>S</mark> VIKNAKRF	KHLLEHDED	EKHLDE	:	49
PfMNPV.Pro	:	MYTRYSYNPSI	GRTYVYDNKYY	KNLG <mark>AVIKNA</mark> NRF	KHFIDHDLD	EKTLDP	:	50
RoMNPV.Pro	:	MPD-YSYRPTI	GRTYVYDNKYY	KNLGSVIKNAKRE	KHLIEHDEE	EKHLDP	:	49
SeMNPV.Pro	:	MYTRYSYNPAI	GRTYVYDNKFY	KNLG <mark>S</mark> VIKNAKRE	EHLIGHEIE	ERTLDE	:	50
SlMNPV.Pro	:	MYTFYSYNPSI	GRTYVYDNKFY	KNLG <mark>S</mark> VIKNAKRF	EHLVHHDID	ERTIDE	:	50
TnSNPV.Pro	:	MYTRYSYSPSI	GRTYVYDNKYY	KNLG <mark>AVIK</mark> NAKRI	KHYADHDLD	BATIDE	:	50
		M Ysy P 0	G4TYVYDNK53	SKNLG 6IKnakr)	kH H2e	E lDp		
		M YSY P (G4TYVYDNK58	SKNLG 6IKnakr)	kH H2 e	E lDp		
		M YSY P 6	G4TYVYDNK53	SKNLG 6IKnakr)	:kH H2 e	E 1Dp 100		
AcMNPV.Pro	:	M YSY P (60 LTN <mark>YLVAEDP</mark> E	G4TYVYDNK55 * T <mark>GPGKNQKL</mark> TI	SKNLG 6IKnakr) 80 .FKEIRNVKPDTME	k H2 e * KLVV <mark>G</mark> WKGKE	E lDp 100 FY <mark>RETW</mark>	:	99
AcMNPV.Pro AgseNPV.Pr	:	M YSY P (60 LDN <mark>YLVAEDPE</mark> LDK <mark>FLVAEDPE</mark>	G4TYVYDNK55 * TGPGKNQKLTI TGPGKNQKLCI	SKNLG GIKnakr) 80 SKEIRNVKPDTME SKEIRNVKPDTME	KLVVGWEGKE	E lDp 100 FY <mark>RETW</mark> FIRETW	:	99 100
AcMNPV.Pro AgseNPV.Pr AngeMNPV.P	::	M YSY P (60 LDN <mark>YLVAEDPE</mark> LDKFLVAEDPE LDH <mark>YIVAEDP</mark> E	G4TYVYDNK55 * TGPGKNQKLTI TGPGKNQKLCI TGPGKNQKLTI	80 80 FKEIRNVKPDTME FKEIRNVKPDTME FKEIRNVKPDTME	KLVV <mark>GWE</mark> GKE KLVV <mark>GWE</mark> GKE KLVVNWSGKE KLIVNWSGKE	E lDp 100 FYRETW FLRETW FLRETW	: : :	99 100 99
AcMNPV.Pro AgseNPV.Pr AngeMNPV.P AnpeMNPV.P	: : :	M YSY P (60 Linylvaedpe Lisflvaedpe Lihylvaedpe Linywvaedpe	SG4TYVYDNK55 * TGPGKNQKLTI TGPGKNQKLCI TGPGKNQKLTI TGPGKNQKLTI	80 80 FKEIRNVKPDTME FKEIRNVKPDTME FKEIRNVKPDTME FKEIRNVKPDTME	KLVV <mark>GWEGKE</mark> KLVVNWSGKE KLVVNWSGKE KLIVNWSGKE	E lDp 100 E <mark>YRETW</mark> FLRETW FLRETW FLRETW		99 100 99 99
ACMNPV.Pro AgseNPV.Pr AngeMNPV.P AnpeMNPV.P ArMNPV.Pro	: : : : : : : : : : : : : : : : : : : :	M YSY P (60 LDNYLVAEDPE LDXFLVAEDPE LDHYIVAEDPE LDNYMVAEDPE LDNYMVAEDPE	SG4TYVYDNK55 * TIGPGKNQKLTI TIGPGKNQKLTI TIGPGKNQKLTI TIGPGKNQKLTI	80 80 FKEIRNVKPDTME FKEIRNVKPDTME FKEIRNVKPDTME FKEIRNVKPDTME FKEIRNVKPDTME	KLUVGWEGKE KLUVGWEGKE KLUVNWSGKE KLIVNWSGKE KLIVNWSGKE KLIVNWSGKE	E lDp 100 FYRETW FIRETW FIRETW FIRETW FIRETW		99 100 99 99
AcMNPV.Pro AgseNPV.Pr AngeMNPV.P AnpeMNPV.P ArMNPV.Pro EmNPV.Pro	: : : : : : :	M YSY P (60 LDNYLVAEDPE LDKFLVAEDPE LDHYIVAEDPE LDNYMVAEDPE LDNYMVAEDPE LDNYMVAEDPE	GG4TYVYDNK55 * TIGPGKNQKLTI TIGPGKNQKLTI TIGPGKNQKLTI TIGPGKNQKLTI TIGPGKNQKLTI	SKNLG 6IKnakr) 80 FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF	KLVV <mark>GWEGKE</mark> KLVVNWSGKE KLIVNWSGKE KLIVNWSGKE KLIVNWSGKE KLIVNWSGKE	E lDp 100 FYRETW FIRETW FIRETW FIRETW FIRETW FIRETW		99 100 99 99 99
AcMNPV.Pro AgseNPV.Pr AngeMNPV.P AnpeMNPV.P ArMNPV.Pro BusNPV.Pro BusNPV.Pro	:::::::::::::::::::::::::::::::::::::::	M YSY P (60 LDNYLVAEDPE LDKFLVAEDPE LDHYIVAEDPE LDNYMVAEDPE LDNYMVAEDPE LDNYMVAEDPE LDNYMVAEDPE	GG4TYVYDNK55 * TGPGKNQKLG TGPGKNQKLT TGPGKNQKLT TGPGKNQKLT TGPGKNQKLT TGPGKNQKLT	80 80 FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF	KLVVEWEGKE KLVVNWSGKE KLIVNWSGKE KLIVNWSGKE KLIVNWSGKE KLIVNWSGKE	E lDp 100 FYRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW		99 100 99 99 99 99
AcMNPV.Pro AgseNPV.Pr AngeMNPV.P AnpeMNPV.P ArMNPV.Pro EmNPV.Pro BusNPV.Pro CfMNPV.Pro	: : : : : : :	M YSY P (GG4TYVYDNK55 * TGPGKNQKLG TGPGKNQKLT TGPGKNQKLT TGPGKNQKLT TGPGKNQKLT TGPGKNQKLT TGPGKNQKLT	80 SKNLG 6IKnakr) SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF	KLVVGWEGKE (LVVNWSGKE (LIVNWSGKE (LIVNWSGKE (LIVNWSGKE (LIVNWSGKE (LIVNWSGKE (LIVNWSGKE	E lDp 100 EYRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW		99 100 99 99 99 100 98
AcMNPV.Pro AgseNPV.Pr AngeMNPV.P AnpeMNPV.Pro EmNPV.Pro BusNPV.Pro CfMNPV.Pro ChchNPV.Pr	: : : : : : : :	M YSY P (60 LD NYLVAEDPE LD KYLVAEDPE LD NYMVAEDPE LD NYMVAEDPE LD NYMVAEDPE LD XYLVAEDPE LD HYMVAEDPE LD HYMVAEDPE LD NYLVAEDPE	* TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI	80 SKNLG 6IKnakr) SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF	KLVV <mark>CWE</mark> GKE (LVVNWSGKE (LIVNWSGKE (LIVNWSGKE (LIVNWSGKE (LIVNWSGKE (LIVNWSGKE (LIVNWSGKE (LIVNWSGKE	E 1Dp 100 FYRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW		99 100 99 99 99 100 98 100
AcMNPV.Pro AgseNPV.Pr AngeMNPV.P AnpeMNPV.Pro EmNPV.Pro BusNPV.Pro CfMNPV.Pro ChchNPV.Pro Choristone		M YSY P (* TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI	80 SKNLG 6IKnakr) SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF	KHH2 KUVV <mark>GWE</mark> GKE KUVVNWSGKE KUVVNWSGKE KUVVNWSGKE KUVVNWSGKE KUVVNWSGKE KUVVNWSGKE KUVVNWSGKE	E 1Dp 100 FYRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW		99 100 99 99 99 100 98 100
AcMNPV.Pro AgseNPV.Pr AngeMNPV.P AnpeMNPV.Pro EmNPV.Pro BusNPV.Pro CfMNPV.Pro ChchNPV.Pro Choristone EcobNPV.Pr		M YSY P (* CGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI	80 FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF	KLVV <mark>GWE</mark> GKE KLVVNWSGKE KLIVNWSGKE KLIVNWSGKE KLIVNWSGKE KLIVNWSGKE KLIVNWSGKE KLIVNWSGKE KLIVNWSGKE KLIVNWSGKE	E 1Dp 100 FYRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW		99 100 99 99 100 98 100 98
ACMNPV.Pro AgseNPV.Pr AngeMNPV.P AnpeMNPV.Pro EmNPV.Pro BusNPV.Pro CfMNPV.Pro ChchNPV.Pr Choristone EcobNPV.Pr		M YSY P (* CGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI	80 SKNLG 6IKnakr) SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF	KH H2 KUVVGWEGKE KUVVWSGKE KUVVWSGKE KUVVWSGKE KUVVWSGKE KUVVWSGKE KUVVWSGKE KUVVWSGKE KUVWSGKE KUVWSGKE	E 1Dp 100 FYRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW		99 100 99 99 100 99 100 99
ACMNPV.Pro AgseNPV.Pr AngeMNPV.P AnpeMNPV.Pro EmNPV.Pro BusNPV.Pro CfMNPV.Pro CfMNPV.Pro Choristone EcobNPV.Pr EppoNPV.Pr HearNPV.Pr		M YSY P (GG4TYVYDNK55 CGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI	80 SKNLG 6IKnakr) SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF SKEIRNVKPDTMF	KH H2 KUVVGWEGKE KUVVNWSGKE KUVVNWSGKE KUVVNWSGKE KUVVNWSGKE KUVVNWSGKE KUVNWSGKE KUVNWSGKE KUVNWSGKE KUVNWSGRE	E 1Dp 100 FYRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW		99 100 99 99 100 100 100 100 100
ACMNPV.Pro AgseNPV.Pr AngeMNPV.P AnpeMNPV.Pro EmNPV.Pro BuSNPV.Pro CfMNPV.Pro ChchNPV.Pro ChchNPV.Pr Choristone EcobNPV.Pr EppoNPV.Pr HearNPV.Pr		M YSY P (60 LDNYLVAEDPE LDKFLVAEDPE LDNYNVAEDPE LDNYNVAEDPE LDNYNVAEDPE LDNYNVAEDPE LDKYLVAEDPE LDRYLVAEDPE LDRYLVAEDPE LDRYLVAEDPE LDRYLVAEDPE LDRYLVAEDPE LDRYLVAEDPE	GG4TYVYDNK55 * TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI	80 80 FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF	KLUVN KLUVNWSGKE KLUVNWSGKE KLUVNWSGKE KLUVNWSGKE KLUVNWSGKE KLUVNWSGKE KLUVNWSGKE KLUVNWSGKE KLUVNWSGKE KLUVNWSGKE	E 100 100 FYRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW		99 100 99 99 99 99 100 98 100 99 100 99
ACMNPV.Pro AgseNPV.Pr AngeMNPV.P AnpeMNPV.Pro EmNPV.Pro CfMNPV.Pro CfMNPV.Pro ChchNPV.Pr Choristone EcobNPV.Pr EppoNPV.Pr HearNPV.Pr HycuNPV.Pro		M YSY P (60 LD NYLVAEDPE LD KFLVAEDPE LD NYNVAEDPE LD NYNVAEDPE LD NYNVAEDPE LD NYNVAEDPE LD YNVAEDPE LD HYMVAEDPE LD HYMVAEDPE LD RYLVAEDPE LD RYLVAEDPE LD RYLVAEDPE LD RYLVAEDPE LD RYLVAEDPE	GG4TYVYDNK55 * TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI	80 SKNLG 6IKnakr) SKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF	KINN H2 € KIVVNWSGKE KIVVNWSGKE KIVNWSGKE KIVNWSGKE KIVNWSGKE KIVNWSGKE KIVNWSGKE KIVNWSGKE KIVNWSGKE KIVVWSGKE KIVVWSGKE KIVVWSGKE	E $1Dp$ 100 FYRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW		99 100 99 99 99 100 99 100 99 100 99 100
ACMNPV.Pro AgseNPV.Pr AngeMNPV.P AnpeMNPV.Pro EmNPV.Pro CfMNPV.Pro ChchNPV.Pro ChchNPV.Pr Choristone EcobNPV.Pr EppoNPV.Pr HearNPV.Pr HycuNPV.Pro MacoNPV-A.		M YSY P (60 LDNYLVAEDPE LDKFLVAEDPE LDNYNVAEDPE LDNYNVAEDPE LDNYNVAEDPE LDNYNVAEDPE LDHYNVAEDPE LDHYNVAEDPE LDHYNVAEDPE LDHYNVAEDPE LDHYNVAEDPE LDRYLVAEDPE LDRYLVAEDPE LDRYLVAEDPE LDRYLVAEDPE LDRYLVAEDPE LDRYLVAEDPE LDRYLVAEDPE	GG4TYVYDNK55 * TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI	80 SKNLG 6IKnakr) SKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF	KLUVN KLUVNWSGKE KLUVNWSGKE KLUVNWSGKE KLUVNWSGKE KLUVNWSGKE KLUVNWSGKE KLUVNWSGKE KLUVNWSGKE KLUVNWSGKE KLUVNWSGKE KLVVNWSGKE	E 1Dp 100 FYRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW		99 100 99 99 99 100 99 90 90 90 90 90 90 90 90 90 90 90 9
ACMNPV.Pro AgseNPV.Pr AngeMNPV.P AnpeMNPV.Pro EmNPV.Pro BusNPV.Pro ChChNPV.Pro ChchNPV.Pro Chcristone EcobNPV.Pr EppoNPV.Pr HearNPV.Pr HycuNPV.Pro MacoNPV-A. MacoNPV-B.		M YSY P (GG4TYVYDNK55 * TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI	80 FKEIRNVKPDTMF	KLVVEWEGKE (LVVWSGKE (LVVWSGKE (LIVNWSGKE (LIVNWSGKE (LIVNWSGKE (LIVNWSGKE (LIVNWSGKE (LIVNWSGKE (LIVNWSGKE (LVVNWSG	E 100 F1 RETW F1 RETW		99 100 99 99 99 99 100 99 100 99 100 99 100 100
AcMNPV.Pro AgseNPV.Pr AngeMNPV.P AnpeMNPV.Pro EmNPV.Pro BusNPV.Pro CfMNPV.Pro ChchNPV.Pro ChchNPV.Pr Choristone EcobNPV.Pr EppoNPV.Pr HearNPV.Pr LdMNPV.Pro MacoNPV-A. MacoNPV-B.		M YSY P (60 LD NYLVAEDPE LD KFLVAEDPE LD NYNVAEDPE LD NYNVAEDPE LD NYNVAEDPE LD NYNVAEDPE LD NYNVAEDPE LD NYLVAEDPE LD RYLVAEDPE LD RYLVAEDPE	GG4TYVYDNK55 TGPGKNQKLT TGPGKNQK TGPGKNQK TGPGKNQK TGPGKNQK TGPGKNQK TGPGKNQK TGPGKNQK TGPGKNQK TGPGKNQK TGPGKNQK TGPGKNQK TGPGKNQK TGPGKNQK TGPGK TG	80 FKEIRNVKPDTMF FK	KLVVGWKGKE KLVVNWSGKE KLVNWSGKE KLVNK KLVNK KLVNWSGKE KLVNWSGKE KLVNK KLVNK	E 1Dp 100 FYRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW		99 100 99 99 99 99 100 99 100 99 100 99 100 100
AcMNPV.Pro AgseNPV.Pr AngeMNPV.P AnpeMNPV.Pro EmNPV.Pro BusNPV.Pro CfMNPV.Pro ChchNPV.Pro ChchNPV.Pr Choristone EcobNPV.Pr EppoNPV.Pr HearNPV.Pr LdMNPV.Pro MacoNPV-A. MacoNPV-B. Maruca_vit OpMNPV.Pro		M YSY P (GG4TYVYDNK55 CGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI TGPGKNQKLTI	80 FKEIRNVKPDTMF FK	KLVVGWEGKE KLVVNWSGKE KLVVNWSGKE KLIVNWSGKE KLIVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVNKSGKE KLVNKS	E $1Dp$ 100 FYRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW		999 100 999 999 108 1099 1009 1009 1000 990
AcMNPV.Pro AgseNPV.Pr AngeMNPV.P AnpeMNPV.Pro EmNPV.Pro BusNPV.Pro CfMNPV.Pro Choristone EcobNPV.Pr Choristone EcobNPV.Pr HearNPV.Pr HearNPV.Pr LdMNPV.Pro MacoNPV-A. MacoNPV-B. Maruca_vit OpMNPV.Pro		M YSY P (* IGPGKNQKLT IGPGKNQK IGP IGP IGP IGP IGP IGP IGP IGP	80 FKEIRNVKPDTMF	KH H2 ● KLVVGWEGKE KLVVNWSGKE KLIVNWSGKE KLIVNWSGKE KLIVNWSGKE KLIVNWSGKE KLIVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE KLVVNWSGKE	E $1Dp$ 100 FYRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW		99 99 99 99 99 100 99 99 100 99 100 100
ACMNPV.Pro AgseNPV.Pr AngeMNPV.P AnpeMNPV.Pro EmNPV.Pro BusNPV.Pro CfMNPV.Pro CfMNPV.Pro Choristone EcobNPV.Pr Choristone EcobNPV.Pr HearNPV.Pr HearNPV.Pr LdMNPV.Pro MacoNPV-A. Maruca_vit OpMNPV.Pro RoMNPV.Pro RoMNPV.Pro		M YSY P (* IGPGKNQKLT IGPGKNQK IGP IGP IGP IGP IGP IGP IGP IGP	80 FKEIRNVKPDTMF FK	KH H2 € KLVVCWSGKE KLVVNWSGKE KLIVNWSGKE KLIVNWSGKE KLIVNWSGKE KLIVNWSGKE KLVVNWSG	E 1Dp 100 FYRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW		99 99 99 99 108 109 109 109 109 100 99 99 90 90 90 90 90 90 90 90 90 90 9
ACMNPV.Pro AgseNPV.Pr AngeMNPV.P AnpeMNPV.P ArMNPV.Pro BusNPV.Pro BusNPV.Pro CfMNPV.Pro Choristone EcobNPV.Pr Choristone EcobNPV.Pr HearNPV.Pr HearNPV.Pr HearNPV.Pro MacoNPV-A. Macuca_vit OpMNPV.Pro PfMNPV.Pro SeMNPV.Pro		M YSY P (60 LD NYLVAEDPE LD NYLVAEDPE LD NYNVAEDPE LD NYNVAEDPE LD NYNVAEDPE LD NYNVAEDPE LD NYNVAEDPE LD NYLVAEDPE LD RYLVAEDPE LD RYLVAEDPE	GG4TYYYDNK55 CG9GKNQKLTI TG9GKNQK TG9GK TG9GKNQK TG9GK TG9GKNQK TG9GK TG9GK TG9G	80 80 FKEIRNVKPDTMF FKEIRNVKP FKEIRNVKPDTMF FKEIRNVKPDTMF FKEIRNVKPDTMF FK	KLUVN GKE (LUVNWS	E 1Dp 100 FYRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW		90 99999999999999999999999999999999999
ACMNPV.Pro AgseNPV.Pr AngeMNPV.P AnpeMNPV.Po EMNPV.Pro BuSNPV.Pro CfMNPV.Pro CfMNPV.Pro Choristone EcobNPV.Pr Choristone EcobNPV.Pr HearNPV.Pr HearNPV.Pr MacoNPV-A. MacoNPV-A. MacoNPV-B. Maruca_vit CpMNPV.Pro SIMNPV.Pro		M YSY P (60 LD NYLVAEDPE LD KFLVAEDPE LD NYNVAEDPE LD NYNVAEDPE LD NYNVAEDPE LD NYNVAEDPE LD NYNVAEDPE LD KYLVAEDPE LD KYLVAEDPE LD KYLVAEDPE LD RYLVAEDPE LD RYLVAEDPE	GG4TYYYDNK55 CG9GKNQKLTI TG9GKNQK TG9GKNQK TG9GKNQK TG9GKNQK TG9GKNQK TG9GKNQK TG9GKNQK TG9GKNQK TG9GKNQK TG9GKNQK TG9GKNQK TG9GKNQK TG9GKNQK TG9GKNQK TG9GKNQK TG9GKNQK	80 FKEIRNVKPDTMF FK	KHHH2 € KUVV@WSGKE KUVVNWSGKE KUVVNWSGKE KUVNWSGKE	E $1Dp$ 100 FYRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW FIRETW		900 999999 1000 10900 10900 10900 10900 10900 10900 1000 10900 1000

Ld 56VAEDPF GPGKNQKLtLFKEIRnVKPDTMKL6VnWsG4EF RETW

		* 120 * 140 *		
AcMNPV.Pro	:	TRFMEDSFPIVNDQEVMDVFLVVNMRPIRPNRCYKFLAQHALR <mark>C</mark> DFDYVP	:	149
AgseNPV.Pr	:	TRFMEDSFPIVNDQEIMDVFLVVNMRP <mark>V</mark> KPNRCYRFLAQHALR <mark>CDF</mark> DYVP	:	150
AngeMNPV.P	:	TREVEDSEPIVNDQEVMDVELVINLRPTRPNRCYKELAQHALRMDCDYVP	:	149
AnpeMNPV.P	:	TREVEDSEPIVNDQEVMDVELVINLRPTRPNRCYKELAQHALRMICJYVP	:	149
Armnpv.pro	÷	TREVEDSEPT VNDQEVMDVELVINLRPTRPNKCYKFLAQHAVRWICDYVP	:	149
BUONDU Dro	:	TREVEDSE FIVINDQEVMDVILVENLAFTRENKCIAFLAQHALAWEDDIVE	:	149
CIMNEN Ero	2	TREMEDSE PLUNDQEIMDUELUUNMRPIRPNRCIRFLAQHALREDENIUP	:	148
ChchNPV Pr	:	TREMEDSEPTIVIDGEVINDVELVVNMRPTRENRCEKFLACHALRCHEDVVE	:	150
Choristone	÷	TREVEDSEPIVNDOEVMDVELVVNMRPTRPNRCYKELAOHALRMDCDYVE		149
EcobNPV.Pr	:	TREMEDSEPIVNDOEVMDVELVINMRPTRPNRCYKFLAOHALRCDEDYVP		150
EppoNPV.Pr	:	TRFVEDSFPIVNDQEVMDVFLVINLRPTRPNRCYKFLAQHALRMCCDYVP	:	149
HearNPV.Pr	:	TRFMEDSFPIVNDQEIMDVFLSVNMRPTKPNRCYRFLAQHALRCDEDYIP	:	150
HycuNPV.Pr	:	TRFMEDSFPIVNDQEVMDVFL ^{VV} NMRPTRPNRCYKFLAQHALR <mark>CD</mark> FDYVP	:	149
LdMNPV.Pro	:	TRFMEDSFPIVNDQEVMDIYL <mark>TINVRPTRPNRCYKFVAQHALR</mark> CDECYVP	:	150
MacoNPV-A.	:	TRFMEDSFPIVNDQEVMDVFLVINMRPTRPNRCYKFLAQHALRCIEDYVP	:	150
MacoNPV-B.	:	TRFMEDSFPIVNDQEVMDVFLVINMRPTRPNRCFKFLAQHALRCDFDYVP	:	150
Maruca_vit	÷	TREVEDSEPTVNDQEVMDVELVVNLRFTRPNRCIKFLAQHALRADODIVP	:	149
DEMNEV.PIC		TREVEDSE PIVNDQEVMDVE UVNMRPIRPNRCI RELAQHALAMBOUIVE	:	192
ROMNEV.Pro	2	TREVEDSEPTVNDQEVNDVELVVNLRPTRENRCYKFLACHALRWDEDYVE	:	149
SeMNPV.Pro	÷	TREMEDSEPTVNDOEIMDVELVINMRPTRPNRCFRFLAGHALRODEDVVE		150
SIMNPV.Pro	÷	TREMEDSFESVNDOEIMDVELVINMRPTRPNRCYRFLAOHALROIEDYVP	-	150
TnSNPV.Pro	:	TRFMEDSFPTVNDQEIMDVFLVVNMRPTRPNRCFKFLAQHALRCIFDYVP	:	150
		TRF6EDSFP1VNDQE6MD65Lv N64Pt4PNRC54F6aQHA6R d dY6P		
		160 * 180 * 200		
AcMNPV.Pro	:	HEVIRIVEPSWVGSNNEYRISLAKKGGGCPIMNLHSEYTNSFEQFIDRVI	:	199
AgseNPV.Pr	:	HEVIRIVEPVYVGNHNEYRISIAKKGGGCPVNNLHSEYTNSFB <mark>E</mark> FINRVI	:	200
AngeMNPV.P	:	HEVIRIVEPSYVEMNNEYRISIAKKEGGCEIMNIHSEYTASFDSFVARVI	:	100 199
Anpemnev.P	÷	HEVIRIVEPSIVEPNNEYRISLAKKGGGCPIMNIHSEYTNSFESFVNRVI	:	100
RIMNEV.PIC	1	HEVIRIVEPSHVGPANEIRISIERAGGGCPIMAIHSEIIASPESPVARVI HEVIRIVEPSHVGPANEIRISIERAGGGCPIMAIHSEIIASPESPVARVI	:	195
BUSNEV Pro	2	HEVIRIVEPSIVGENNEYRISLAKEGGCCPVMNLHSEYTNSFFFFTNEVT	:	200
CfMNPV.Pro	÷	HEVIRIVEPSYVCMNNEYRISLAKKGGGCPIMNIHAEYTNSFFSFVNRVI		198
ChchNPV.Pr	÷	HEVIRIVEPSWVG <mark>SNNEYRISLAKKGGGCPIMNLHSEYTNSFE</mark> FI <mark>A</mark> RVI	:	200
Choristone	:	HEVIRIVEPSYVC <mark>M</mark> NNEYRISLAKKGGGCPIMNIH <mark>A</mark> EYTNSFE <mark>S</mark> FVNRVI	:	199
EcobNPV.Pr	:	HEVIRIVEPSYVC <mark>S</mark> NNEYRISLAKRGGGCPVMNLH <mark>A</mark> EYTNSFE <mark>E</mark> FINRVI	: .	200
EppoNPV.Pr	:	HEVIRIVEPSYVG <mark>MNNEYRISLAKKG</mark> GGCPIMNIHSEYTNSFE <mark>S</mark> FVNRVI	:	199
HearNPV.Pr	:	HEVIRIVEPSYVGSNNEYRISLAKK <mark>Y</mark> GGCPVMNLHEFYTNSFE <mark>F</mark> FI EN VI	:	200
HycuNPV.Pr	:	HEVIRIVEPSWVCSNNEYRISLAKKGGGCPIMNLHSEYTNSFEQFIDRVI	:	199
LdMNPV.Pro	:	HEVIRIVEPS-TVENNEYRISLAKRGGGCPIRNLHSAYTTSFEHFLNSVI	:	199
MacoNPV-A.	÷	HEVIRIVEPSYVCSNNEYRVSLAKRGGGCPVMNLHSEYTNSFDDFINRVI	:	200
Maconpv-B. Maruga wit	÷	HEVIRIVEPSIVGSNNEIRVSLAARGGGCPVMNLHSEIINSFEFFINRVI		100
OpMNEV Pro	1	HEVIRIVEPSIVGPANEIRISLAKKGGGCPIMNIHSEIINSPEIFVNRVI HEVIRIVEPSIVGPANEIRISLAKKGGGCPIMNIHSEIINSPEIFVNRVI	:	100
PfMNPV.Pro	÷	HEVIRIVEPSIVEPINEIRISLARREGGCPUMNIHEEIRISFEEFURVI HEVIRIVEPSIVESNNEYRVSLARREGGCPUMNIHEEYTNSFEFFURVI	:	200
RoMNPV.Pro	÷	HEVIRIVEPSYVCANNEYRISLAKKGGGCPIMNIHSEYTNSFESFVSRVI		199
SeMNPV.Pro	:	HEVIRIVEP <mark>V</mark> YVG <mark>INNEYRISLAKKGGGCPVMNLHSEYTNSFE</mark> FINRVI	: :	200
SlMNPV.Pro	:	HEVIRIVEPSYVC <mark>S</mark> NNEYRISLAKKGGGCPVMNLHSEYT <mark>H</mark> SFE <mark>E</mark> FINRVI	:	200
TnSNPV.Pro	:	HEVIRIVEPSWVG <mark>S</mark> NNEYRISLAKKGGGCPIMNLHSEYTNSFE <mark>E</mark> FINRVI	: 3	200
		HeVIRIVEPs vg nNEYR6SLaK4gGGCP6mN6HseYTnSFE F6 rVI		
		* 220 * 240		
AcMNPV.Pro	:	WENFYKPIVYIGTDSAEEEEILLEVSLVFKVKEFAPDAPLF <mark>I</mark> GPAY :	2	45
AgseNPV.Pr	:	WENFYKPIVYIGTDS ^A EEEEILLELSLVFKIKEFAPDAPLY <mark>N</mark> GPAY :	2	46
AngeMNPV.P	:	WENFYKPIVYIGTDSGEEEEILIEVSLVFKVKEFAPDAPLFIGPAY :	2	45
AnpeMNPV.P	:	WENFYKPIVYIGTDSCEEEEILIEVSLVFKVKEFAPDAPLF¶GPAY :	2	45
ArMNPV.Pro	:	WENFYKPIVYIGTDSCEEEEILIEVSLVFKVKEFVPDAPLFIGPAY :	2	45
EmNPV.Pro	:	WENFYKPIVYIGTDSÆEEEILIEVSLVFKIKEFAPDAPLFIGPAY :	2	45
BusNPV.Pro	:	WENFYKPIVYVGTDSAEEEEILLEVSLVFKVKEFAPDAPLYTGPAY :	2	46
CfMNPV.Pro	:	WENFYKPIVYIGTDSGEEEEMLIEVSLVFKVKEFAPDAPLFIGPAY :	2	44
ChchNPV Pr	:	WENFYKPIVYVGTUSAEEEEEEEVSLVFKIKEFAPDAPLYSGPAY :	2	46
Choristone	:	WENFYKPIVYIGTUSGEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	2	45
ECODNPV.Pr	:	WENFYKELVYVGTUSAEEEEIILLEVSLVFKIKEFAPDAELYSGPAY :	2	46
LppoNPV.Pr	÷	WENTIALLYILGTD SCEEELLLEVSLVFKVKEFAPDAPLFHGPAY :	2	45
HEALNPY.Pr	÷	WENFIKELVIVGTUSAEEEEILLEVSLIFKIKEFAPDAPLYMGPAY :	2	40
nycunev.er	:	WEDFINFLVIIGTUSAEEEEILLEVSLVFKVKEFAPDAPDFTGPAY :	2	40
MacoNDV 7	•	WENEYKDIVYVCTDSAFFFFFFFFVATVFFFFAPDAFDFQGPAY	2	40
MacoNDV-P		WENEYKDIVYVCTD SAFFFFFTTTFVSIVFNTKEFAPDAFDINGPAI:	2	46
Maruca vit	:	WENEYKDIVYICTDSCEFFFILIEVSLVFKUKFFADDADI TECDAY	- 2	45
OpMNEV Pro	;	WENFYKPIVYIGTDSSEFEFILIEVSLVEKVKEFADDADI SCOAY	2	45
PfMNPV.Pro	÷	WENFYKPIVYVGTDSAEEEEILLEVSLVFKIKFFAPDAPLYNGPAY	2	46
RoMNPV.Pro	:	WENFYKPIVYIGTDSGEEEEILIEVSLVFKVKEFAPDAPLPTGPAY	2	45
SeMNPV.Pro	:	WENFYKPIVYVGTDSGEEEEILLELSLVFKIKEFAPDAPLYNGPAY	2	46
SlMNPV.Pro	:	WENFYKPIVYVGTDSGEEEEILLEVALVFKIKEFAPDASLYNGPAY :	2	46
TnSNPV.Pro	:	WENFYKPIVYVGTDSAEEEEILLEVSLVFKIKEFAPDAELYSGPAY :	2	46
		We1FYKPIVY6GTdS EEEE6L6E6sL6FK6KEFaPDApL5 GPAY		

Figure 2. Alignment of the polyhedrin genes of baculoviruses, The sequences were aligned using DNAstar CLUSTAL W program.



Figure 3. Phylogeny of the polyhedrin protein. Phylogenetic tree of polyhedrin gene was constructed by MEGA version 3.1 from CLUSTAL W alignments. The neighbor-joining method was used to construct the tree. From the phylogenetic tree, the polh gene of AnpeNPV was closest to that of ArNPV.



Figure 4. The secondary structure of the Polh protein of AnpeNPV. It contains 4 regions of alpha helix and 11 pieces of *B*-sheet.

of helical and 11 pieces of β -sheet in the sequence.

3.3. Construction of Expression Plasmid

The fragment of polh-Ap was sequenced to be sure containing a correct ORF, and was inserted into the expression pET28a vector and then was expressed in *E col* (BL21) The recombinant plasmid was identified by digestion with *Eco*R I and *Hind* III. The result of electrophoresis indicated the recombinant plasmid was successfully constructed (Figure 6).

3.4. Expression of the AnpeNPV *polh* Gene in *E. coli*

The *E. coli* BL21 transformed with the pET28a/polh-Ap plasmid to express the His-6PGL fusion protein of about 34 kDa, which was consistent with the expected mo lecular mass of the fusion protein of pET28a/polh-Ap (Figure 7). The result showed that the AnpeNPV *polh* gene was highly expressed in *E. coli*. The expression products can be used as antigen to raise the antibody of the Polh protein.

SciRes Copyright © 2009



Figure 5. The hydrophobicity profile of AnpeNPV Polh protein. The X-axis contains 245 increments, each representing an amino acid in the sequence of AnpeNPV polyhedrin. The Y-axis represents the range of hydrophilicity values (from 2.2 to -3.1) with employ of Kyte-Doolittle scale. One region of strongly hydrophilicity exists at positions from 30 to 50 of the AnpeNPV polyhedrin protein.



Figure 6. Identification of the recombinant plasmid pET28a/polh-Ap by electrophoresis in agarose gel 1, pET28a/polh-Ap; M, DNA molecular mass marker.



Figure 7. The expression products of AnpeNPV polh gene were analyzed by SDS-PAGE and Western-blot 1, Protein of E. coli BL21 contained pET28a induced by IPTG; 2, Protein of E. coli BL21 contained pET28a /polh-Ap induced by IPTG; 3, Western-blot of the fusion protein; M, Protein marker.

4. DISCUSSION

In this report the AnpeNPV *poly* gene was cloned and compared with other baculoviruses. Polyhedrin genes

SciRes Copyright © 2009



Figure 8. Mass-spectrum with Mascot analysis (Mass: 29003; Score: 107; Expect: 0.00013) Protein score is -10*Log (P), where P is the probability that the observed match is a random event. Protein scores greater than 81 are significant (p<0.05).

are highly conserved among many baculoviruses. The AnpeNPV polyhedrin gene was closest to that of ArNPV from the Phylogeny tree (Figure 3), differing by only five amino acids (Figure 2). DNA sequence comparison polyhedra containing low numbers of virions [16]. of AnpeNPV and ArNPV polyhedrins showed that a difference in identity to 97%, of which only thirteen differences (Figure 2). The result suggests that the *poly* genes of AnpeNPV and ArNPV have evolved from a common ancestor distinct from the other NPVs. The AnpeNPV *polh* gene is very closely related to NPV group I than that of group II (Figure 3). Availability of polyhedrin protein sequences of other baculoviruses may aid in their classification and may help define baculovirus species [14].

Alignment results showed that the variability regions occur at the beginning of N-terminus (position 2 to 4) and the domain from position 31 to 52 (Figure 2). Even in this region, some positions are conserved, such as, H37, H41, E45 and D49. In contrast, the C-terminus (from 198 to 245) is highly conserved. The cysteine positons (at 133 and 179) and the prolines (at 60, 64, 81, 109, 127, 130, 150, 159, 180, 207, 236, and 244) of AnpeNPV polyhedrin appear to be very important (Figure 2). Cysteines often form disulphide bonds critical for protein structure; proline breaks helical and β -sheet regions and is often associated with turns in the secondary structure of proteins [15]. Therefore, both these amino acids could be crucial in determining the conformation of these proteins. These conserved regions may be necessary to give the proteins their characteristic common properties: namely crystal formation and alkali solubility. Indeed, a mutant of AcNPV with a single pro tein changed to Leu at position 62 resulted in cubic polyhedra containing low numbers of virions [16].

Hydrophilic regions are exposed on surface of the protein and are highly polar. They have a tendency to be antigenic sites [17]. There is one region of strongly hydrophilicity at positions from 30 to 50 in the AnpeNPV polyhedrin (**Figure 5**). Comparison of the baculovirus polyhedrin sequences indicates that although they vary in amino acid sequence in this region, their basic pattern of hydrophilicity is preserved (date no shown). Therefore, much of the variation in amino acid sequence is neutral and does not alter the overall nature of the proteins. This region therefore presents a potential antigenic site which may be useful for production of antibodies capable of differentiating or identifying different baculoviruses [18]. Ultimately predicted antigenic determinants from proteins of pathogenic organisms might also be useful in the production of synthetic vaccines [17].

The *polh* gene of baculovirus is a very late gene which expressed in late stage of virus infection. It is not an essential gene in virion development and could be deleted for foreign gene expression [19,20]. Some evidences showed that the level of the foreign gene expression was related to genetic codes of the gene. To test the *polh* gene expression in another system, we constructed a bacterium expression system to express the Polh protein. The result indicates that this gene is suitable for *E. coli* expression system. It might be helpful to produce the virus proteins to raise antibodies.

ACKNOWLEDGEMENTS

This work was supported by the 973 National Basic Research Program of China (2005CB121005); The Six-Field Top programs of Jiangsu Provice; National Natural Science Foundation of Jiangsu Education Communitte(06KJD180043); Innovation Foundation for Graduate Students of Jiangsu Province.

REFERENCES

- Q. Fan, S. Li, L. Wang, B. Zhang, B. Ye, Z. Zhao, Cui, L. (2007). The genome sequence of the multinucleocapsid nucleopolyhedrovirus of the Chinese oak silkworm Antheraea pernyi. Virology 366(2), 304-315.
- [2] O.A. Lihoradova, I. D. Ogay, A. A. Abdukarimov, S. S. Azimova, D. E. Lynn, Slack, J. M. (2007). The Homingbac baculovirus cloning system: An alternative way to introduce foreign DNA into baculovirus genomes. J Virol Methods 140 (1-2), 59–65.
- [3] Z. M. Nie, Z. F. Zhang, D. Wang, P. A. He, C. Y. Jiang, L. Song, F. Chen, J. Xu, L. Yang, L. L. Yu, J.Chen, Z. B. Lv, J. J. Lu, X. F. Wu, Zhang Y. Z. (2007) Complete sequence and organization of Antheraea pernyi nucleopolyhedrovirus, a dr-rich baculovirus. BMC Genomics 8, 248–261.
- [4] S. P. Cook, R. E. Webb, J. D. Podgwaite, Reardon, R. C. (2003) Increased mortality of gypsy moth Lymantria dispar (L.) (Lepidoptera: Lymantriidae) exposed to gypsy moth nuclear polyhedrosis virus in combination with the phenolic gycoside salicin. J Econ Entomol 96(6), 1662–1667.
- [5] Moscardi, F. (1999) Assessment of the application of baculoviruses for control of Lepidoptera. Annu Rev Entomol 44,

257-289

- [6] X. Dai, T. M. Stewart, J. A. Pathakamuri, Q. Li, Theilmann, D. A. (2004) Autographa californica multiple nucleopolyhedrovirus exon0 (orf141), which encodes a RING finger protein, is required for efficient production of budded virus. J Virol 78(18), 9633–9644.
- [7] S. G. Kamita, S. Maeda, Hammock, B. D. (2003) High-frequency homologous recombination between baculoviruses involves DNA replication. J Virol 77(24), 13053–13061.
- [8] A. M. Khurad, A. Mahulikar, M. K. Rathod, M. M. Rai, S. Kanginakudru, Nagaraju J. (2004) Vertical transmission of nucleopolyhedrovirus in the silkworm, Bombyx mori L. Journal of Invertebrate Pathology 87, 8–15.
- [9] S. Gomi, C. E. Zhou, W. Y. Yih, K. Majima, Maeda S. (1997) Deletion analysis of four of eighteen late gene expression factor gene homologues of the baculovirus, BmNPV. Virology 230, 35-47.
- [10] W. B. Wang, S. Y. Zhu, L. Q. Wang, F. Yu, Shen W. D. (2005) Cloning and sequence analysis of the Antheraea pernyi nucleopolyhedrovirus gp64 gene. J Biosci 30, 605–610.
- [11] L. H. Robert, Bonning B.C. (2003) Comparative analysis of the genomes of Rachiplusia ou and Autographa californica multiple nucleopolyhedroviruses Journal of General Virology 84, 1827–1842.
- [12] S. Gomi, K. Majima, Maeda S. (1999) Sequence analysis of the genome of Bombyx mori nucleopolyhedrovirus. J Gen. Virol. 80, 1323–1337.
- [13] M. D. Ayres, S. C. Howard, J. Kuzio, M. Lopez-Ferber, Possee R.D. (1994) The complete DNA sequence of Autographa californica nuclear polyhedrosis virus. Virology 202, 586-605.
- [14] E. A. van Strien, D. Zuidema, R.W. Goldbach, Vlak, J. M. (1992) Nucleotide sequence and transcriptional analysis of the polyhedrin gene of Spodoptera exigua nuclear polyhedrosis virus. J Gen Virol 73 (Pt 11), 2813–2821.
- [15] P. Y. Chou, Fasman, G. D. (1977) Beta-turns in proteins. J Mol Biol 115(2), 135–175.
- [16] E. B. Carstens, A. Krebs, Gallerneault, C. E. (1986) Identification of an amino acid essential to the normal assembly of Autographa californica nuclear polyhedrosis virus polyhedra. J Virol 58(2), 684–688.
- [17] T. P. Hopp, Woods, K. R. (1981). Prediction of protein antigenic determinants from amino acid sequences. Proc Natl Acad Sci U S A 78(6), 3824-3828.
- [18] Rohrmann, G. F. (1986) Polyhedrin structure. J Gen Virol 67(8), 1499–1513.
- [19] R. D. Possee, S. C. Howard, (1987) Analysis of the polyhedrin gene promoter of the Autographa californica nuclear polyhedrosis virus. Nucleic Acids Res 15(24), 10233-10248.
- [20] G. E. Smith, M. J. Fraser, Summers, M. D. (1983) Molecular engineering of the Autographa californica nuclear polyhedrosis virus genome:deletion mutations within the polyhedrin gene. J Virol 46, 584–593.